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(54) [Title of Invention] PLASMID PNC903

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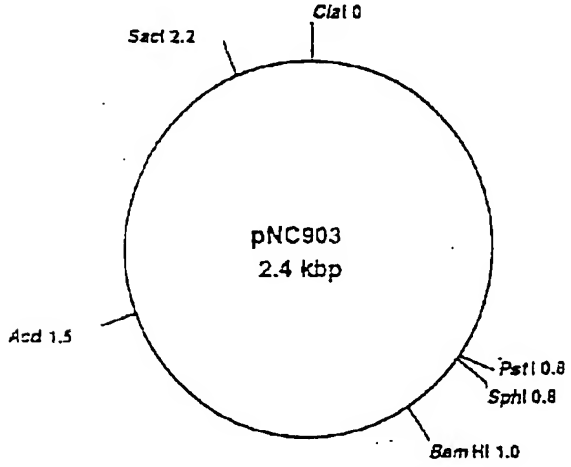
(57)【要約】

【構成】 分子量が約2.4kbの環状プラスミドであり、制限酵素 EcoRI、 XbaI、 KpnI、 HindIII、 BglII、又は SpeI によって切断されず、制限酵素 ClaI、PstI、 SphI、 BamHI、 AccI、及び SacI によって切断される部位数がそれぞれ１であり、且つ前記の制限酵素 ClaI、 PstI、 SphI、 BamHI、 AccI、及び SacI によって切断される部位が図１の制限酵素開裂地図で示されるプラスミドpNC903。

【効果】 環状プラスミドpNC903は、それに含まれる種々の制限酵素による開裂部位を利用して、外来のＤＮＡ断片を導入修飾し、多くの有用なプラスミドベクターの開発に利用でき、特に、ノカルディオフォルム細菌を宿主とする宿主－ベクター系におけるプラスミドベクターとして有用である。



【特許請求の範囲】

【請求項１】 分子量が約2.4kbの環状プラスミドであり、制限酵素 EcoRI、 XbaI、 KpnI、 HindIII、 BglII、又は SpeI によって切断されず、制限酵素 ClaI、 PstI、 SphI、 BamHI、 AccI、及び SacI によって切断される部位数がそれぞれ１であり、且つ前記の制限酵素 ClaI、 PstI、 SphI、 BamHI、 AccI、及び SacI によって切断される部位が下記の制限酵素開裂地図化１で示されるプラスミドpNC903。

(74) [Attorney(s) Representing All Applicants]

[Patent Attorney]

(57) [Abstract]

[Constitution] Molecular weight being ring shape plasmid of approximately 2.4 kb, to be, restriction enzyme EcoRI, XbaI, KpnI, HindIII, BglII, Or quantity of site which is not cut off by SpeI, is cut offby restriction enzyme ClaI, PstI, SphI, BamHI, AccI, and SacI is 1 respectively, plasmid pNC903 where sitewhich is cut off and aforementioned restriction enzyme ClaI, by PstI,the SphI, BamHI, AccI, and SacI is shown withthe restriction enzyme cleavage map of Figure 1.

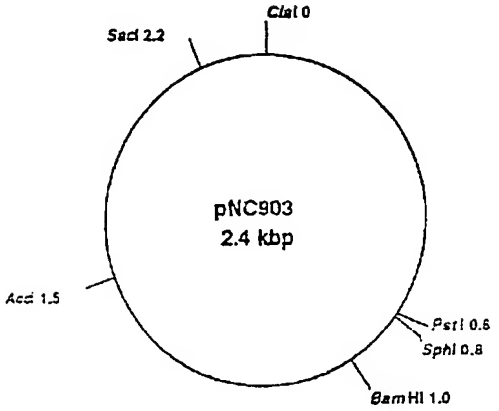
[Effect(s)] Ring shape plasmid pNC903 be able to introduce and be able to decorate importedDNA fragment, be able to utilize in development of many useful plasmid vector making useof cleavage site due to various restriction enzyme which is included in that, especially,it is useful as plasmid vector in host-vector system which designates no cull D. offお jp11ム bacteria as host.

[Claim(s)]

[Claim 1] Molecular weight being ring shape plasmid of approximately 2.4 kb, to be, restriction enzyme EcoRI, XbaI, KpnI, HindIII, BglII, Or quantity of site which is not cut off by SpeI, is cut offby restriction enzyme ClaI, PstI, SphI, BamHI, AccI, and SacI is 1 respectively, plasmid pNC903 where sitewhich is cut off and aforementioned restriction enzyme ClaI, by PstI,the SphI, BamHI, AccI, and SacI is shown withthe below-mentioned restriction enzyme cleavage map Chemical Formula 1.

【化１】

[Chemical Formula 1]



【請求項２】　該環状プラスミドが、Rhodococcus rhodochrous P-II-123-1(工業技術院生命工学工業技術研究所 寄託番号 FERM P-14193) 由来のプラスミドであることを特徴とする請求項１に記載のプラスミドpNC903。

[Claim 2]　Said ring shape plasmid, plasmid pNC903 which is stated in Claim 1 which designatesthat it is a plasmid of Rhodococcus rhodochrous P-II-12 3-1(Agency of Industrial Science and Technology National Institute of Bioscience and Human-Technology deposit number FERM P-14193) derivation as feature.

【発明の詳細な説明】

[Description of the Invention]

【０００１】

[0001]

【産業上の利用分野】本発明は、新規なプラスミドに関し、具体的には、エポキシド生産菌であるRhodococcus属に属する微生物に由来する新規な環状プラスミドに関する。本発明の環状プラスミドは、それに含まれる制限酵素によって切断される部位を利用して、両端に当該制限酵素によって切断されるＤＮＡ配列を有するＤＮＡ断片を連結してなる人工の環状プラスミドの調製に用いることができる。

[Field of Industrial Application] This invention regards novel ring shape plasmid which derives in microorganism which belongsto Rhodococcus being attached which concretely, is a epoxide producing microbe in regard tothe novel plasmid. Making use of site which is cut off by restriction enzyme which isincluded in that, connecting DNA fragment which possesses DNA sequence whichis cut off to both ends by this said restriction enzyme you can use for manufacturing theartificial ring shape plasmid which becomes ring shape plasmid of this invention.

【０００２】

[0002]

【従来の技術】短鎖長オレフィンを炭素源として、土壌中より分離したノカルディア属、ロドコッカス属といったノカルディオフォルム細菌はオレフィンを酸化して、対応するエポキシド類を生産することが知られている。例えば、プロピレンを炭素源として土壌中より分離したノカルディア・コラリーナは、光学活性なエポキシドや、合成樹脂、医薬、農薬などの有機化学製品の製造原料中間体として広範囲に利用できるトリフルオロプロペンオキシド（TFPO)の生産などに利用されている。これらの土壌中より分離したノカルディオフォルム細菌を用い、更なる菌株改良のため、エポキシド生産能を有するノカルディオフォルム細菌の宿主ーベクター系の開発が以前から期待されていた。これらの微生物を宿主とするに適したベクター開発は遅

[Prior Art] With short chain long olefin as carbon sourc e, as for no cull D. off お jp11ム bacteria such as Nocardiaceae and Rhodococcus sp. which are separated from inthe soil oxidation doing olefin, it is known that it produces theepoxide which correspond. Nocardia * コ rally ナ which is separated from in soil with the for example propylene as carbon source is utilized production etc of trifluoro propene oxide (TFPO) which canbe utilized in broad range in optically active epoxide and synthetic resin, pharmaceutical and asthe process raw material intermediate of pesticide or other organic chemical

れているが、本発明者は、既にエポキシド生産株のノカルディア・コラリーナより、ノカルディオフォルム細菌の宿主ーベクター系に適用可能なプラスミドpNC500を見いだし、特許出願した（特開 平 5-244953 号公報を参照）。また、ロドコッカス属の一部を宿主とする、宿主ーベクター系の開発例として、ジャーナル オブ バクテリオロジー(J.Bacteriol.)170,638(1988)、アプライドアンド エンバイロメンタル マイクロバイオロジー(Appl.Environ.Microbiol.)56,2818(1990)、プラスミド(Plasmid)23,242(1990) 等の数例が報告されている。

product. Making use of no cull D. off お jp11 ム bacteria which is separated fromin soil of these, for strain improvement on that of ,development of host-vector system of no cull D. off お jp11 ム bacteria whichpossesses epoxide production ability was expected from time before. Designates these microorganism as host vector development which issuited is late, but this inventor, from Nocardia * コ rally ナ of theepoxide producing strain, you discovered applicable plasmid pNC500 already in host-vector system of no cull D. offお jp11 ム bacteria, patent application did (Japan Unexamined Patent Publication Hei 5-244953 disclosure reference). In addition, portion of Rhodococcus sp. is designated as host, the journal of bacteriology (Journal of Bacteriology (0021-9193, JOBAAY))170,638(1988) and Applied and en bi- Ro mentha jp11 microbiology (Applied and Environmental Microbiology (0099-2240, AEMIDF))56,2818(1990), plasmid (Plasmid)23,242(1990) or other several examples is reported as developmentexample of host-vector system

【０００３】しかしながら、前記するプラスミドpNC500を除き、従来より報告されているノカルディオフォルム細菌由来のプラスミドの多くは、ロドコッカス属の一部のみを宿主にするにすぎないものであった。そのため、より広い範囲のノカルディオフォルム細菌を宿主として適用でき、宿主のエポキシド生産菌から、微生物を育種、改良するための新しいベクターの開発が強く要望されている。

[0003] But, many of plasmid of no cull D. off お jp11 ム bacteria derivationwhich is reported from until recently excluding plasmid pNC500 whichdescription above is done, only designate only portion of the Rhodococcus sp. as host were something. Because of that, be able to apply no cull D. off お jp11 ム bacteria, of awider range, as host from epoxide producing microbe of host, in orderbreeding, to improve microorganism development of new vector isstrongly demanded.

【０００４】

【発明が解決しようとする課題】本発明は上記の課題を解決するもので、本発明の目的は、ノカルディオフォルム細菌を宿主として、ノカルディオフォルム細菌中において複写が可能である新規な環状プラスミドを提供することにある。特には、ノカルディオフォルム細菌に類別されるロドコッカス属に属する微生物に由来する新規な環状プラスミドＤＮＡを提供することにある。

[0004]

[Problems to be Solved by the Invention] As for this in vention being something which solves above-mentionedproblem, objective of this invention is to offer novel ring shape plasmid where copy ispossible with no cull D. off お jp11 ム bacteria as host, in in theno cull D. off お jp11 ム bacteria. Especially, it is to offer novel ring shape plasmid DNA which derives in microorganism whichbelongs to Rhodococcus sp. which classifies in no cull D. off お jp11 ムbacteria.

【０００５】

【課題を解決するための手段】本発明者らは、ノカルディオフォルム細菌を宿主とでき、ＤＮＡ組換えに使用可能な新規なプラスミドＤＮＡを開発すべく鋭意研究を行ったところ、当該細菌に類別され、エポキシド生産菌であるロドコッカス属に属する微生物から、宿主ーベクター系におけるベクターとして利用可能な新規な環状プラスミドを見出し、本発明を完成した。

[0005]

[Means to Solve the Problems] As for these inventors, no cull D. off お jp11 ム bacteria in order that it canmake host, develops useable novel plasmid DNA in DNA rearrangement when thediligent research was done, was classified in this said bacteria, from microorganism whichbelongs to Rhodococcus sp. which is a epoxide producing microbe, practical novel ring shape plasmid was discovered as thevector in host-vector system, this invention was completed.

【０００６】本発明のプラスミドは、分子量が約 2.4 kb の環

[0006] As for plasmid of this invention, molecular wei

状プラスミドであり、制限酵素 EcoRI、 XbaI、 KpnI、 HindIII、 BglII、又は SpeI によって切断されず、制限酵素 ClaI、 PstI、 SphI、 BamHI、 AccI、及び SacI によって切断される部位数がそれぞれ１であり、且つ前記の制限酵素 ClaI、 PstI、 SphI、 BamHI、 AccI、及び SacI によって切断される部位が下記の制限酵素開裂地図化１で示されるプラスミドであり、該プラスミドにプラスミドpNC903の呼称を本発明者は付与する。

ght being ring shape plasmid of approximately 2.4 kb , to be, restriction enzyme EcoR1 , XbaI, KpnI, HindIII, BglII, Or it is cut off by SpeI , restriction enzyme ClaI, PstI, Quantity of site which is cut off by SphI, BamHI, the AccI , and SacI is 1 respectively, is plasmidwhere site which is cut off and aforementionedrestriction enzyme ClaI, by PstI, SphI, BamHI, AccI , andthe SacI is shown with below-mentioned restriction enzyme cleavage map Chemical Formula 1, naming plasmid pNC903 itgrants this inventor to said plasmid.

【化１】

[Chemical Formula 1]

【０００７】本発明のプラスミドpNC903を保有する微生物として、具体的にロドコッカス属に属する微生物であり、プロピレン資化性菌として土壌から分離した、エポキシド生産能を有する菌株 Rhodococcus rhodochrous P-II-123-1(工業技術院生命工学工業技術研究所 寄託番号 FERM P-14193) が挙げられる。なお、この微生物Rhodococcus rhodochrous P-II-123-1 は、上記の寄託番号 FERM P-14193 で通産省工業技術院生命工学工業技術研究所に寄託されており、また、該菌株の菌学的性質は表１に示すとおりである。

[0007] It was a microorganism which concretely belongs to Rhodococcus sp. as microorganism which possesses plasmid pNC903 of this invention, you can list strain Rhodococcus rhodochrous P-II-12 3-1(Agency of Industrial Science and Technology National Institute of Bioscience and Human-Technology deposit number FERM P-14193) whichit separated from soil as propylene-feeding microbe, possesses epoxide production ability. Furthermore , this microorganism Rhodococcus rhodochrous P-II-12 3- 1 with above-mentioned deposit number FERM P-14193 thedeposit is done in Ministry of International Trade and Industry Agency of Industrial Science and Technology National Institute of Bioscience and Human-Technology, in addition, microbiological characteristic of said strain is asshown in Table 1.

【０００８】

[0008]

【表１】

[Table 1]

菌学的性質		
グラム染色性		+
胞子		−
運動性		−
形状		桿菌
コロニーの形状		ピンクオレンジ、不透明、円形、規則性、凸状、直径 0.5㎜以下 (3日間培養)
各温度での生育	37℃	+
	45℃	(+)
カタラーゼ		+
オキシダーゼ		−
OFテスト		−

【０００９】細胞の化学分析

[0009] Chemical analysis of cell

ミコール酸を含む。細胞壁のジアミノ酸はメソ-DAPである。脂肪酸成分は、16:0, 16:1, 18:1, 10-Me・18:0及び微量の14:0, 15:0, 17:0から成っている。

Mycolic acid is included. diamino acid of cell wall is meso-DAP. aliphatic acid component has consisted of 14:0,15:0,17:0 of 16:0,16:1,18:1,10-Me * 18:0 and

trace amount.

【００１０】生化学テスト

[0010] Biochemistry test

分解	Disassembly
アデニン +	Adenine +
チロシン +	Tyrosine +
尿素 −	Urea -
単一炭素源での生育	Growth with single carbon source
イノシトール[1] −	Inositol 1 -
マルトース[1] (+)	Maltose 1 (+)
マンニトール[1] +	Mannitol 1 +
ラムノース[1] −	Rhamnose 1 -
ソルビトール[1] +	Sorbitol 1 +
m−ヒドロキシ安息香酸[2] +	M - hydroxybenzoic acid 2 +
アジピン酸塩[2] +	Adipate 2 +
安息香酸塩[2] +	Benzoate 2 +
クエン酸塩[2] +	Citrate 2 +
乳酸塩[2] +	Lactate 2 +
グルタミン酸塩[2] −	Glutamate 2 -
L-チロシン[2] +	L- tyrosine 2 +
グリセロール[1] (+)	Glycerol 1 (+)
トレハロース[1] (+)	Trehalose 1 (+)
p-ヒドロキシ安息香酸[2] +	P- hydroxybenzoic acid 2 +
D-マンノース[1] −	D- mannose 1 -
アセトアミド[2] +	Acetamide 2 +
D-ガラクトース[1] −	D- galactose 1 -
酵素活性	Enzyme activity
α−グルコシダーゼ +	- glucosidase +
システインアリルアミダーゼ −	Cysteine allyl amidase -
バリンアリルアミダーゼ −	Valine allyl amidase -
生育テスト（存在下）	Growth test (Under of existence)
5% NaCl +	5% NaCl +

アジドナトリウム (0.02% w/v) －

【００１１】注: 表中の肩表記する記号は、培地に対する添加濃度を示し、また、符号(+)は下記の水準を意味する。

【００１２】1. 1 %(w/v)

2. 0.1 %(w/v)

(+) weak posotive

【００１３】本発明のプラスミドpNC903は、具体的には上記微生物 Rhodococcus rhodochrous P-II-123-1 から、次に示す方法により分離することができる。

【００１４】ノカルディオフォルム細菌は、通常の培地中で培養するとき、リゾチームなどの溶菌酵素で溶菌されにくいので、予めグリシン入り培地で当該微生物を培養し、ペニシリンGで処理することにより、溶菌酵素で溶菌され易い菌を得る。例えば、下記の組成のＮＢＧ培地にグリシンを１%(w/v)程度添加した培地が用いられる。

（ＮＢＧ培地）

Nutrient Broth No.2 (Oxoid)	２．５ %(w/v)
グルコース	１．０ %(w/v)

当該微生物を３０℃で対数増殖後期まで培養し、更にペニシリンG所定量を培地に添加し数時間培養を継続した後、溶菌酵素（リゾチーム、アクロモバクターペプチターゼ 等）で溶菌する。得られる溶菌液より、環状プラスミドＤＮＡは、公知の方法で分離することができる (Biochimca. et Biophysica. Acta., 383, 457-463 (1975) を参照）。更に、分離された環状プラスミドＤＮＡの集合から、アガロースゲル上で電気泳動することにより、分子量の異なる環状プラスミドＤＮＡを分離し、目的の分子量を有する環状プラスミドＤＮＡを単離することができる。なお、当該微生物 Rhodococcus rhodochrous P-II-123-1 は、環状プラスミドＤＮＡとして、分子量が約 2.4 kb の該プラスミドpNC903 一種のみを有しており、容易に単離することができる。

【００１５】分子量が約 2.4 kb の環状プラスミドとして単離される当該プラスミドpNC903の有する種々の制限酵素に対する感受性を調べると、その感受性は表２に示すものである。

Azido sodium (0.02% w/v) -

[0011] Note: Shoulder notation of in the table symbol which is done shows theaddition concentration for culture medium, in addition, symbol (+) means below-mentionedlevel.

[0012] 1. 1 %(w/v)

2. 0.1 %(w/v)

() Weak posotive

[0013] It can separate plasmid pNC903 of this inventio n, due to method which fromthe above-mentioned microorganism Rhodococcus rhodochrous P-II-12 3-1 , is shown concretely next.

[0014] Because no cull D. off お jp11 ム bacteria is dif ficult to be done, whenculturing in conventional culture medium, lysis with lysozyme or other bacteriolytic enzyme, this said microorganism iscultured beforehand with glycine entering culture medium, microbe which thelysis is easy to be done is obtained with bacteriolytic enzyme by treating withthe pennicilin G. 1 % (w/v) extent is added culture medium which can use glycine for NBG culture medium ofthe for example below-mentioned composition.

(NBG culture medium)

Nutrient Broth No.2 (Oxoid)	2.5 % (w/v)
Glucose	1.0 % (w/v)

With 30 °C it cultures this said microorganism to logar ithmic proliferation post phase, furthermore addsthe pennicilin G predetermined amount to culture medium and after continuing several hours culture, lysisit does with bacteriolytic enzyme (Such as lysozyme and Achrombacter ペプ jp8 tar ゼ). fromlysate which is acquired, it can separate cyclic plasmid DNA,with known method (Bi ochimca. et Bi ophysica. Acta., 383, 457-463 (1975) reference). Furthermore, cyclic plasmid DNA where molecular weight differs from gathering of thecyclic plasmid DNA which is separated, by electrophoresis doing on agarose gel, can beseparated, cyclic plasmid DNA which possesses molecular weight of object can beisolated. Furthermore , as for this said microorganism Rhodococcus rhodochrous P-II-12 3- 1 , molecular weight has had only thesaid plasmid pNC903 one kind of approximately 2.4 kb as cyclic plasmid DNA, can isolate easily.

[0015] When molecular weight sensitivity for various r estriction enzyme which this said plasmid pNC903 which isisolated as ring shape plasmid of

approximately 2.4 kb has is inspected,
thesensitivity is something which is shown in Table 2.

【００１６】

【表２】

プラスミドpNC903の制限酵素に対する感受性

制限酵素	切断個所の数
EcoRI	0
XbaI	0
KpnI	0
HindIII	0
BglII	0
SpeI	0
ClaI	1
PstI	1
SphI	1
BamHI	1
AccI	1
SacI	1

【００１７】なお、上記制限酵素は、それぞれ次の菌種から得られる制限酵素である。

制限酵素	菌種の名称
EcoRI	Escherichia coli RY13
XbaI	Xanthomonas badrii (ATCC 11672)
KpnI	Klebsiella pneumoniae OK8
HindIII	Haemophilus influenzae Rd
BglII	Bacillus globigii
SpeI	Sphaerotilus natans (ATCC 13923)
ClaI	Caryophanon latum L
PstI	Providencia stuartii 1641 pPst101
SphI	Streptomyces phaeochromogenes

[0016]

[Table 2]

It confronts restriction enzyme of plasmid pNC903 sen sitivity

Number of restriction enzyme	cutting site
EcoR1	0
XbaI	0
KpnI	0
HindIII	0
BglII	0
SpeI	0
ClaI	1
PstI	1
SphI	1
BamHI	1
AccI	1
SacI	1

[0017] Furthermore, above-mentioned restriction enzym e is restriction enzyme which is acquiredfrom respective following microbe type.

Title of restriction enzyme	microbe type
EcoR1	Escherichia coli RY13
XbaI	Xanthomonas badrii (ATCC 11672)
KpnI	Klebsiella pneumoniae OK8
HindIII	Haemophilu sin fluenzae Rd
BglII	Bacillus globigii
SpeI	Sp ha erotilus n ata ns (ATCC 13923)
ClaI	Ca ryop ha non latu ml
PstI	Providencia stuartii 1641 pPst101
SphI	Streptomyces phaeochromogenes

BamHI	Bacillus subtilis MT-2 (pBamHI RM22)
AccI	Acinetobacter calcoaceticus
SacI	Streptomyces achromogenes (ATCC 12767)

【００１８】各制限酵素による切断個所の数は、過剰量の制限酵素存在下にプラスミドpNC903を完全消化し、得られる消化物（切断されたＤＮＡ）を０．７%アガロースゲル電気泳動にかけ、分離可能なＤＮＡ断片の種類数から決定される。大腸菌のラムダファージのＤＮＡ(λＤＮＡ)を制限酵素 HindIII で消化し得られる、既知分子量のＤＮＡ断片(J. Mol. Biol., 98, 551-564 (1975)を参照)を同一のアガロースゲル上での電気泳動にかけ、その泳動距離で描かれる標準曲線に基づき、切断されたＤＮＡ断片の分子量は算出した。上記の制限酵素 ClaI、PstI、SphI、BamHI、AccI、又は SacI で完全消化し、得られる消化物（切断されたＤＮＡ）は、それぞれ約 2.4 kb の分子量を有する単一のＤＮＡ断片であった。更に、該プラスミドpNC903を、上記の制限酵素 ClaI、PstI、SphI、BamHI、AccI、SacI の２種以上を組み合わせて用いた処理によって得られる、複数のＤＮＡ断片の分子量をそれぞれアガロースゲル電気泳動で測定することにより、図１に示す制限酵素開裂地図を作成した。なお、互いに近接して存在する PstIと SphIの開裂位置は、アガロースゲル上での電気泳動による分子量の測定では容易に区別できないが、PstIと BamHIにより切断される約 0.2 kb ＤＮＡ断片中に SphIの切断部位が存在することを確認することで PstIと SphIの開裂位置の関係を特定した。また、本発明のプラスミドpNC903は、その由来するノカルディオフォルム細菌（Rhodococcus rhodochrous P-II-123-1）中において複製が行え、ノカルディオフォルム細菌のＤＮＡ複製開始点（レプリコン）を内在している。

【００１９】本発明のプラスミドpNC903は、図１に示す制限酵素による開裂部位を有しており、該開裂部位を利用して、該プラスミドを修飾して種々の有用なプラスミドベクターを開発することができる。更には、該プラスミド或はその修飾により得られる誘導体に、目的とする遺伝子のＤＮＡ断片、例えば、モノオキシゲナーゼ等の酵素或は蛋白質をコードする遺伝子のＤＮＡ断片を上記の制限酵素による開裂部位を利用して組み込み、得られる環状プラスミドを宿主微生物に導入して、宿主を形質転換させることが可能である。当該プラスミドpNC903を宿主ーベクター系として適用できる宿主微生物としては、該プラスミドpNC903が由来する Rhodococcus rhodochrous P-II-123-1 と類似するＤＮＡ鎖合成酵素（ＤＮＡポリメラーゼ）などプ

BamHI	Bacillus subtilis MT-2 (pBamHI RM22)
AccI	Acinetobacter calc oacceticus
SacI	Streptomyces ac hr omogenes (ATCC 12767)

[0018] Plasmid pNC903 complete digestion it does quantity of cutting site dueto each restriction enzyme, under restriction enzyme existing of excess quantity, it appliesthe digest (It was cut off DNA) which is acquired on 0.7 % agarose gel electrophoresis, is decided from thequantity of types of separable DNA fragment. digestion it does DNA (DNA) of lambda phages of E. coli with restriction enzyme HindIII and is acquired, you applied DNA fragment (Journal of Molecular Biology (0022-2836, JMOBAK) , 98, 55 1-564 (1975) reference) of known molecular weight on theelectrophoresis on same agarose gel, you calculated molecular weight of DNA fragment whichis cut off on basis of standard curve which is drawn with phoresis distance. Above-mentioned restriction enzyme ClaI, complete digestion it did with PstI,the SphI, BamHI, AccI or SacI , digest (It was cut off DNA)which is acquired was single DNA fragment which possesses molecular weight of therespective approximately 2.4 kb . Furthermore, restriction enzyme cleavage map which is shown in Figure 1 by being acquiredby treatment which is used said plasmid pNC903, above-mentioned restriction enzyme ClaI,combining 2 kinds or more of PstI, SphI, BamHI, AccI andthe SacI measures molecular weight of DNA fragment of multiple respectivelywith agarose gel electrophoresis, was drawn up. Furthermore , proximity doing mutually, cleavage position of the PstI and SphI which exist cannot distinguish with themeasurement of molecular weight with electrophoresis on agarose gel easily, but itwas related cleavage position of PstI and SphI by factthat you verify that cleavage site of SphI exists in approximately 0.2 kb DNA fragment which is cut off by PstI and BamHI specific. In addition, as for plasmid pNC903 of this invention, duplication does, theDNA replication starting point (replicon) of no cull D. off お jp11 ム bacteria is indwelling that in in theno cull D. off お jp11 ム bacteria (Rhodococcus rhodochrous P-II-12 3- 1) which derives.

[0019] Plasmid pNC903 of this invention cleavage site due to restriction enzyme which is shown inthe Figure 1 has had, making use of said cleavage site, decorating said plasmid, thevarious useful plasmid vector can develop. Furthermore, in derivative which is acquired said plasmid or by thedecoration, DNA fragment of gene which is made object, to installthe DNA fragment of gene which for example monooxygenase or other enzyme or protein code is donemaking use of cleavage site due to above-mentioned restriction enzyme, introducing cyclic plasmid which is acquired into host microorganism,

ラスミドＤＮＡの複製を司る酵素群を有するノカルディオフォルム細菌が好適に用いられる。なお、該プラスミドpNC903を宿主ーベクター系として適用するに際しては、該プラスミドを汎用される選択マーカーの遺伝子、例えば、放線菌由来のチオストレプトン耐性遺伝子、大腸菌由来のクロラムフェニコール耐性遺伝子、アンピシリン耐性遺伝子、カナマイシン耐性遺伝子などの薬剤耐性をもたらす遺伝子ＤＮＡにより修飾して、耐性遺伝子を内在したプラスミドベクターとして用いると好ましい。即ち、該プラスミドpNC903を修飾して得られる耐性遺伝子を内在したプラスミドベクターに目的の遺伝子のＤＮＡ断片を組み込み得られる環状プラスミドを作製し、この環状プラスミドを目的とする宿主微生物に導入して、前記の薬剤耐性を指標とし選別することで、容易に該環状プラスミドにより形質転換された微生物を選別することができる。

neoplastic transformation the host it is possible to do. It can use for ideal no cull D. off お jp11 ム bacteria which possessesthe enzyme group which administers duplication of plasmid DNA such as theDNA chain synthase (DNA polymerase) which resembles Rhodococcus rhodochrous P-II-12 3- 1 where said plasmid pNC903 derives as host-vector systemthe this said plasmid pNC903 as host microorganism which it can apply. Furthermore , when said plasmid pNC903 when it applies, as host-vector systemdecorating gene of selectable marker which said plasmid is widely used, thethio ス pick-up プ ton resistance gene of for example Actinomycetes derivation, chloramphenicol resistance gene of E. coli derivation, with genetic DNA which brings ampicillin resistance gene and kanamycin resistance gene or other drug resistance, ituses resistance gene as plasmid vector which is indwelling, it is desirable. Namely, decorating said plasmid pNC903, resistance gene which is acquired it producesthe cyclic plasmid which installs DNA fragment of gene of object in theplasmid vector which is indwelling, is acquired, introducing into host microorganismwhich designates this cyclic plasmid as object, it designates theaforementioned drug resistance as indicator and by fact that it sorts,easily it can sort microorganism which neoplastic transformation is done due to said cyclic plasmid .

【００２０】なお、上記の該プラスミドpNC903の耐性遺伝子による修飾、或はポリペプチドや蛋白質をコードする遺伝子などのＤＮＡ断片の組み込みは、公知の遺伝子ＤＮＡ組み換え技術、例えば、参照文献 Scientific American 233(1) 24-33 (1975)、 Molecular Cloning : a Laboratory Manual. Cold Spring Harbor, NY : Cold Spring Harbor (1982) などに記載されている手法を用いて行うことができ、目的とする環状プラスミドはその分子量を量り分取することができる。また、本発明のプラスミドpNC903、或は該プラスミドpNC903から上記のＤＮＡ組み換え技術により調製される環状プラスミドを宿主微生物に導入するには、例えば、宿主微生物であるノカルディオフォルム細菌の細胞をプロトプラスト或はスフェロプラストにして、環状プラスミドをポリエチレングリコール（ＰＥＧ）と共存させることで環状プラスミドを移入する方法（J. Bacteriol. 170, 638-645 (1988)などを参照）、または細胞を対数増殖期中期まで培養し、高電圧の電気パルスを与えて環状プラスミドを移入するエレクトロポレーション法（Appl. Enviro. Microbio. 56, 2818-2825 (1990) などを参照）等の公知の技術を適用できる。

[0020] Furthermore, as for installation of gene or other DNA fragment which decoration orthe polypeptide and protein due to resistance gene of above-mentioned said plasmid pNC903the code is done, it is possible to do genetic DNA rearranging technology ofthe public knowledge, making use of technique which is stated in for example cited reference Scientific American 233(1) 2 4- 33 (1975) andthe Molecular Cloning : a Laboratory Manual. Cold Spring Harbor , NY : Cold Spring Harbor (1982) etc cyclic plasmid which is made objective measures molecular weight andthe fraction collection is possible. In addition, plasmid pNC903 of this invention, Or cyclic plasmid which is manufactured from said plasmid pNC903 by theabove-mentioned DNA rearranging technology is introduced into host microorganism, cyclic plasmid method which imports cyclic plasmid by fact that itcoexists with polyethylene glycol (PEG) (Journal of Bacteriology (0021-9193, JOBAAY) 170, 638-645 (1988) etc reference), or cell is cultured to logarithmic growth phase midperiod thecell of no cull D. off お jp11 ム bacteria which is a for example host microorganism to protoplastor spheroplast, electric pulse of high voltage is given and electroporation method (Ap pl . Enviro. Microbio. 56, 2818-2825 (1990) etc reference) or other known technologywhich imports cyclic plasmid can be applied.

【００２１】更には、本発明のプラスミドpNC903をプラスミドベクターとして用い、目的とする酵素或は蛋白質をコードする

[0021] Furthermore, With plasmid pNC903 of this inv ention as plasmid vector to use, It is possible to

遺伝子を組み込み得られる環状プラスミドを導入した宿主微生物の形質転換株は、それ自体を公知の方法で培養して、目的とする酵素或は蛋白質を産出させることができ、又目的とする酵素とその基質との反応による有用な代謝物の製造を行うことができる。即ち、目的とする酵素或は蛋白質を細胞外に分泌生産させたり、或は細胞内に生産蓄積させたりでき、更には生産させた酵素により、例えば、基質のオレフィンを酵素により酸化して、対応する光学活性なエポキシド類を生産するなどの酵素反応を行ったり、難分解物質を酵素反応により分解して代謝物に変換したりすることができる。

produce useful metabolite with enzyme which is made the objective or to install gene which protein code is done as forthe neoplastic transformation strain of host microorganism which introduces cyclic plasmid which is acquired,culturing that itself with known method, it it makes objective of theenzyme or it to be possible fact that it produces protein, withthe reaction of enzyme and substrate which in addition it makes theobjective. Namely, enzyme or, protein secretory production doing or product accumulation be ableto do, furthermore with enzyme which is produced in extracellular,, theolefin of for example substrate oxidation doing with enzyme , or other enzymatic reaction whichproduces optically active epoxide which correspond to do, difficultly digested matter quality which is madethe objective disassembling with enzymatic reaction , it can convert to metabolite inthe intracellular.

【００２２】

[0022]

【実施例１】

[Working Example 1]

Rhodococcus rhodochrous P-II-123-1 からのプラスミドpNC903の単離

Isolation of plasmid pNC903 from Rhodococcus rhodochrous P-II-123-1

Rhodococcus rhodochrous P-II-123-1 のスラントより３白金耳量を採取し、それを液体培地〔Nutrient Broth No.2(Oxoid) 2.5 %(w/v), グルコース 1 %(w/v), グリシン 1 %(w/v)〕 500ml に接種し、30℃で21時間振盪培養した。この時点で、前記培養液中における濃度が 0.5 units/ml となる量のペニシリンGを添加し、さらに30℃で3時間培養を継続した。次いで、培養した当該菌の菌体を該培養液から集菌し、TE緩衝液〔0.025M トリス（ヒドロキシメチル）アミノメタン（トリス）、0.025M EDTA : pH8.0〕で洗浄した。該菌体を、溶菌液〔0.3Mショ糖、0.025M トリス、0.025M EDTA、2 mg/ml リゾチーム、2 mg/ml アクロモペプチダーゼ、50 μg/ml RNase : pH8.0〕20ml 中に懸濁し、30℃で2時間反応させた。得られる反応液に、2%(w/v)ラウリル硫酸ナトリウムと0.3M水酸化ナトリウムからなる溶液10mlを添加し、良く混合してから55℃の湯浴中に１時間置いた。この液に、フェノール・クロロホルム（１容：１容）混合液 4ml を加え、１分間良く混ぜ、液全体を白濁させた。

3 platinum loop quantity it recovered from slant of Rhodococcus rhodochrous P-II-123-1 , that theinoculation did in liquid culture medium (Nutrient Broth No.2(Oxoid) 2.5 % (w/v), glucose 1 % (w/v), glycine 1 % (w/v)) 500 ml , 2 1 hour shaking culture did with 30 °C. With this time point, pennicilin G of quantity where concentration in theaforementioned culture medium becomes 0.5 units/ml was added, furthermore 3 hoursculture continued with 30 °C. Next, microbe collection it did cell mass of this said microbe which was cultured fromthe said culture fluid, washed with TE buffer (0.025M tris (hydroxymethyl) aminomethane (tris) and 0.025 MED TA : pH 8.0). Suspension it did said cell mass, in lysate (0.3M sucrose , 0.025M tris , 0.025 MED TA,2 mg/ml lysozyme , 2 mg/ml achromopeptidase (EC 3.4.21.50) and 50 g/ml RNase : pH 8.0) 20 ml , 2 hours reacted with 30 °C. in reaction mixture which is acquired, to add solution 10 ml whichconsists of 2 % (w/v) sodium lauryl sulfate and 0.3M sodium hydroxide, after mixing well, 1 hour youput in warm bath of 55 °C. In this liquid, 1 minute it mixed well including phenol * chloroform (1 permitting/inserting:1 permitting/inserting) mixed solution 4 ml ,the clouding did total liquid.

【００２３】この白濁した液を、4℃で30分間17000×ｇの遠心分離にかけ、得られる上層の溶液を分取した。再度、それと等量のフェノール・クロロホルム（１容：１容）混合液を加え良く混ぜた後、4℃で30分間17000×ｇの遠心分離にかけ、分離した上層の溶液を分取した。

[0023] Liquid which this clouding is done, was applied on centrifugal separation of the30-minute 17000 X g with 4 °C, solution of top layer which isacquired fraction collection was done. For second time, after mixing well that and including phenol * chloroform (1 permitting/inserting:1 permitting/inserting) mixed solution of equivalent, you applied on centrifugal

separation of 3 0-minute 17000 X g with the4 ℃, fraction collection you did solution of top layer which is separated.

【００２４】この分取した上層の溶液に、それと等量のジエチルエーテルを加え、穏やかに撹拌した後しばらく放置した。分離する上層のジエチルエーテルを捨て、再度下層の溶液に、それと等量のジエチルエーテルを加え抽出した。前記のエーテル抽出後、得られる下層の液に、0.1倍容の3M 酢酸ナトリウム水溶液及び2倍容のエタノールを加え、析出した沈殿物を遠心分離で回収した。回収した沈殿物を5mlのTE緩衝液に溶解し、更に CsCl 7.5g と、1.5mg/ml 臭化エチジウム-TE緩衝液2ml とを加え混合し、溶液を得た。この溶液を42時間 120,000×gの密度勾配遠心分離にかけた。

[0024] In solution of top layer which this fraction colle ction is done, after agitatingcalmly, that and including diethyl ether of equivalent, it left for awhile. You threw away diethyl ether of top layer which it separates, for thesecond time in solution of bottom layer, you extracted that andincluding diethyl ether of equivalent. After aforementioned ether extraction, in liquid of bottom layerwhich is acquired, precipitate which was precipitated including the 0.1 time permitting/inserting 3M sodium acetate aqueous solution and 2-foldpermitting/inserting ethanol, it recovered with centrifugal separation. it melted precipitate which recovers in TE buffer of the5 ml, it mixed furthermore including with CsCl 7.5g and 1.5 mg/ml ethidium bromide-TE buffer 2 ml ,acquired solution. This solution was applied on density gradient centrifugal separation of 4 2 hours 120,000 X g.

【００２５】遠心分離したプラスミド画分を、紫外線照射により検出し、分取した。分取したプラスミド画分を、n-ブタノールで処理し臭化エチジウムを除いた。その後、TE緩衝液に対して透析し、エタノール沈殿により精製プラスミド画分を得た。該精製プラスミド画分を 0.5 mlのTE緩衝液に溶解し、0.7%アガロースゲル電気泳動(100 V、 1時間)に供し、分子量 約 2.4 kb のプラスミドバンドの存在を確認した。該分子量 約 2.4kb のプラスミドバンドを含むゲル部分を分取した。このゲル部分を少量のTE緩衝液とともに透析膜に入れ、電気泳動させ、更に逆方向に短時間泳動させた後、透析膜内に残る溶液を回収し、この回収した溶液に、0.1倍容の3M 酢酸ナトリウム水溶液及び2倍容のエタノールを加え、析出した沈殿物を遠心分離で回収して、真空乾燥を行い、目的のプラスミドpNC903を得ることができる。なお、上記の精製プラスミド画分をアガロースゲル電気泳動に供し、分子量の異なるプラスミドを分離する際、前記分子量 約 2.4kb のプラスミドバンド以外のプラスミドバンドは見出されず、当該菌の有する環状プラスミドは、プラスミドpNC903のみであることが判る。

[0025] Centrifugal separation it detected plasmid fractio n which is done, with ultraviolet light illumination, the fraction collection did. plasmid fraction which fraction collection is done, was treated with n- butanol and theethidium bromide was excluded. dialysis it did after that, vis-a-vis TE buffer, itacquired refining plasmid fraction with ethanol precipitation. It melted said refining plasmid fraction in TE buffer of 0.5 ml, offeredto 0.7% agarose gel electrophoresis (100 V and 1 hour), verified existence of plasmid band of molecular weight approximately 2.4 kb . gel portion which includes plasmid band of said molecular weight approximately 2.4 kb the fraction collection was done. With TE buffer of trace inserting this gel portion in dialysis membrane, the electrophoresis doing, furthermore with centrifugal separation solution which in reverse directionremains after short time phoresis and inside dialysis membrane it recovers, this in thesolution which recovers, precipitate which it precipitated including the 0.1 time permitting/inserting 3M sodium acetate aqueous solution and 2-foldpermitting/inserting ethanol, recovering, vacuum drying it does, plasmid pNC903of objective can acquire. Furthermore , above-mentioned refining plasmid fraction is offeredto agarose gel electrophoresis, when separating plasmid where molecular weight differs, theplasmid band other than plasmid band of aforementioned molecular weight approximately 2.4 kb is not discovered, as for cyclic plasmid which this said microbe has, onlythe plasmid pNC903 it understands that is.

【００２６】また、プラスミドpNC903を、各種制限酵素によって単一消化、二重消化、或いは三重消化し、得られるＤＮＡ断片をアガロースゲル電気泳動にかけ、各ＤＮＡ断片の移動度から分子量を求めたところ、プラスミドpNC903の分子量は約 2.4 kb であった。この場合、制限酵素の反応条件は、供給者に

[0026] In addition, plasmid pNC903, when single dig estion, double digestion or triple digestionit did with various restriction enzyme, it applied DNA fragmentwhich is acquired on agarose gel electrophoresis, sought molecular weight from mobility

よって定められた条件に従った。なお、分子量は、λDNAを制限酵素 HindIII で分解して得られる断片の標準移動度のパターンを基にして決定した。更に、これらの結果から、該プラスミドpNC903は図１の制限酵素地図を示すことが分かった。また、アガロースゲル上での電気泳動による分子量の測定では容易に区別できない、互いに近接して存在する PstIと SphIの開裂位置は、 PstIと BamHIにより切断される約 0.2 kbＤＮＡ断片中に SphIの切断部位が存在することを確認することで PstIと SphIの開裂位置の関係を特定した。

ofeach DNA fragment, molecular weight of plasmid pNC903 was approximately 2.4 kb . In this case, reaction condition of restriction enzyme, you followed condition which isdecided by supplier. Furthermore , disassembling DNA with restriction enzyme HindIII , thepattern of standard mobility of fragment which is acquired it decided the molecular weight, on basis of. Furthermore, from these results, as for said plasmid pNC903 it understood thatthe restriction enzyme map of Figure 1 is shown. In addition, you carinot distinguish with measurement of molecular weightwith electrophoresis on agarose gel easily, proximity doing mutually, it waslocated PstI and SphI which exist cleavage, by fact thatyou verify that cleavage site of SphI exists in approximately 0.2 kb DNA fragmentwhich is cut off by PstI and BamHI relationship of cleavageposition of PstI and SphI specific.

【００２７】

[0027]

【発明の効果】本発明の環状プラスミドpNC903は、種々の制限酵素による開裂部位各一つを有しており、この特定される開裂部位を利用して、外来のＤＮＡ断片を導入修飾し、多くの有用なプラスミドベクターを開発することができる。更に、当該プラスミドは、ノカルディオフォルム細菌を宿主として、ノカルディオフォルム細菌中において複写が可能である、宿主ーベクター系におけるプラスミドベクターとして有用である。また、該プラスミドを用いて、外来のＤＮＡ断片を導入修飾して得られる環状プラスミドベクターとし、更に得られる環状プラスミドベクターに種々の酵素或は蛋白質をコードする遺伝子を組み込み得られる環状プラスミドを導入した宿主微生物の形質転換株を利用し、該形質転換株を培養して目的の酵素或は蛋白質を産出させることができ、又目的とする酵素とその基質との反応による有用な代謝物や酵素反応産物の製造を行うことができる。

[Effects of the Invention] Cyclic plasmid pNC903 of th is invention has had cleavage site each one due to various restriction enzyme,making use of cleavage site which this specific is done, can introduce candecorate imported DNA fragment, can develop many useful plasmid vector. Furthermore, as for this said plasmid, copy is possible with no cull D. offお jp11ム bacteria as host, in in no cull D. offお jp11ム bacteria,it is useful as plasmid vector in host-vector system. In addition, said plasmid using, Introducing decorating imported DNA fragment, cyclic plasmid vector which is acquiredto do, Furthermore it is possible to produce useful metabolite and enzymatic reaction product with thevarious enzyme or to install gene which protein code is done makinguse of neoplastic transformation strain of host microorganism which introduces cyclic plasmid which isacquired, culturing said neoplastic transformation strain, enzyme of objective or it ispossible, to produce protein, with reaction of enzyme and thesubstrate which in addition it makes objective in cyclic plasmid vector which isacquired.

【図面の簡単な説明】

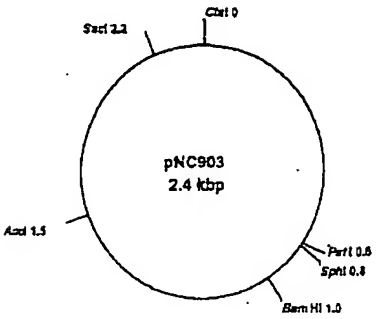
[Brief Explanation of the Drawing(s)]

【図１】 制限酵素 ClaI、PstI、SphI、BamHI、AccI、及びSacIによる切断部位の相対位置を示すプラスミドpNC903の制限酵素開裂地図。

[Figure 1] Restriction enzyme cleavage map of plasmid pNC903 which shows relative position of cleavage site due to therestriction enzyme ClaI, PstI, SphI, BamHI, AccI , and theSacI.

【図１】

[Figure 1]



4

```
DDDDD   UU  UU     444  555555    666    00000
 DD DD  UU  UU    4444  55       66     00   00
 DD  DD UU  UU   44 44  55555   66      00  000
 DD  DD UU  UU  44  44      55  66666   00 0000
 DD  DD UU  UU  4444444     55  66  66  0000 00
 DD DD  UU  UU      44  55  55  66  66  000  00
DDDDD   UUUUUU     4444  5555    6666    00000
```

```
 2222    00000   00000
22  22  00   00 00   00
    22  00  000 00  000
  222   00 0000 00 0000
 22     0000 00 0000 00
22  22  000  00 000  00
222222   00000   00000
```

02/13/2002

Use of SYCO for catalyst optimization

SYCO is a series of related plasmids in which operons relevant for 3G production are expressed by promoters of varying strengths on a single plasmid. The operons may include, but are not limited to, *dhaB1-3,X*, *orfY,X,W*, and *dar1/gpp2*. Individual operons may also be complete or may be missing one or more of the genes that make up the operon. The promoters include, but are not limited to, the GI promoter mutants previously described. The various expression cassettes (promoter-operon) are modular and can be assembled in a wide variety of configurations. This allows direct analysis of the impact of expression level on catalyst performance, and therefore facilitates catalyst optimization.

EXAMPLE
Use of SYCO to examine effect of orf expression on catalyst performance

The orf operon from pKP32 (described previously) was PCR amplified (SEQ ID NOS: 6-7) with *Hind*III at the 5' end and *Avr*II at the 3' end, and subcloned between *Hind*III and *Avr*II in pLitmus28 (New England Biolabs) to generate pKP38. The *EcoRI/Hind*III restriction fragment containing the GI mutant promoter P1.6 from pMP38/1.6 (described elsewhere) was subcloned between *EcoR*I and *Hind*III in pKP38 to generate pKP39. The *Avr*II/*Xba*I restriction fragment containing the *dhaB* expression cassette from pMP38/1.6 was subcloned between *Avr*II and *Xba*I in pLitmus28 (New England Biolabs) to generate pMP39. The *Avr*II/*Xba*I restriction fragment containing the *dhaB* expression cassette from pMP39 was subcloned into the *Avr*II site of pRJ50 to generate pSYCO11. The *Avr*II restriction fragment containing the orf expression cassette from pKP39 was subcloned into the *Nhe*I site of pSYCO11 to generate pSYCO12. The plasmids pSYCO11 and pSYCO12 are identical except that pSYCO11 does not contain the orf operon. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of orf expression on catalyst performance metrics such as titer (Table 1), rate, yield or B12 utilization.

Table 1

	orf expression	3G titer (g/L)
pSYCO11	no	58
pSYCO12	yes	110

EXAMPLE
Use of SYCO to examine effect of orf expression level on catalyst performance

The *EcoRI/Hind*III restriction fragment containing the GI mutant promoter GI P1.5 from pMP38/1.5 (described elsewhere) was subcloned between *EcoR*I and *Hind*III in pKP38 to generate pKP40. The *Avr*II restriction fragment containing the orf operon driven by GI P1.5 from pKP40 was subcloned into the *Nhe*I site of pSYCO11 to generate pSYCO13. The *EcoRI/Hind*III restriction fragment containing the GI mutant promoter P1.20 from pMP38/1.20 was subcloned between *EcoR*I and *Hind*III in pKP38 to generate pKP41.

The *Avr*II restriction fragment containing the orf expression cassette from pKP41 was subcloned into the *Nhe*I site of pSYCO11 to generate pSYCO14. The *EcoR*I/*Hind*III restriction fragment containing the GI mutant promoter P3.4 from pMP38/3.4 was subcloned between *EcoR*I and *Hind*III in pKP38 to generate pKP47. The *Avr*II restriction fragment containing the orf expression cassette from pKP47 was subcloned into the *Nhe*I site of pSYCO11 to generate pSYCO24. The plasmids pSYCO12, pSYCO13, pSYCO14 and pSYCO24 are identical except for the specific GI promoter mutant used to express the orf operon, which is P1.6, P1.5, P1.20 and P3.4, respectively. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of orf expression level on catalyst performance metrics such as titer (Table 2), rate, yield or B12 utilization.

Table 2

	relative orf expression	3G titer (g/L)
pSYCO12	1.0	110
pSYCO13	0.25	80
pSYCO14	0.0625	95
pSYCO24	6.6	88

EXAMPLE

Use of SYCO to examine effect of *orfY* expression on catalyst performance

The *orfX-orfW* genes were PCR amplified using primers designed to incorporate a *Hind*III site at the 5' end and an *Avr*II site at the 3' end (SEQ ID NOS: 1-2) from pKP32 and the product was subcloned between *Hind*III and *Avr*II in pLitmus28 (New England Biolabs) to generate pKP37. The *EcoR*I/*Hind*III restriction fragment containing the GI mutant promoter P1.6 from pMP38/1.6 was subcloned between *EcoR*I and *Hind*III in pKP37 to generate pKP43. The *Avr*II restriction fragment containing P1.6 expressing *orfX-orfW* from pKP43 was subcloned at *Nhe*I in pSYCO11 to generate pSYCO15. The *EcoR*I/*Hind*III restriction fragment containing the GI mutant promoter P1.5 from pMP38/1.was subcloned between *EcoR*I and *Hind*III in pKP37 to generate pKP44. The *Avr*II restriction fragment containing P1.5 expressing *orfX-orfW* from pKP44 was subcloned at *Nhe*I in pSYCO11 to generate pSYCO16. The *EcoR*I/*Hind*III restriction fragment containing the GI mutant promoter P1.20 from pMP38/1.20 was subcloned between *EcoR*I and *Hind*III in pKP37 to generate pKP45. The *Avr*II restriction fragment containing P1.20 expressing *orfX-orfW* from pKP45 was subcloned at *Nhe*I in pSYCO11 to generate pSYCO17. The *EcoR*I/*Hind*III restriction fragment containing the GI mutant promoter P3.4 from pMP38/3.4 was subcloned between *EcoR*I and *Hind*III in pKP37 to generate pKP48. The *Avr*II restriction fragment containing P3.4 expressing *orfX-orfW* from pKP48 was subcloned at *Nhe*I in pSYCO11 to generate pSYCO25. The plasmids pSYCO12 and pSYCO15 are identical except that pSYCO15 does not express *orfY*. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of *orfY* expression on catalyst performance metrics such as titer (Table 3), rate, yield or B12 utilization.

Table 3

	orfY expression	3G titer (g/L)
pSYCO12	yes	110
pSYCO15	no	122

SEQ ID NO: 1
CAT AAG CTT AAC GAG GGG ACC GTC ATG TCG

SEQ ID NO: 2
ACG CCT AGG CCA GTT CAA GCG CAA GCA TCA G

EXAMPLE
Use of SYCO to examine effect of glycerol dehydratase expression level on catalyst performance

The *Avr*II/*Not*I restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO12 was replaced with the corresponding *Avr*II/*Not*I restriction fragment from pMP38/1.5 to generate pSYCO18. The *Avr*II/*Not*I restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO13 was replaced with the corresponding *Avr*II/*Not*I restriction fragment from pMP38/1.5 to generate pSYCO19. The *Avr*II/*Not*I restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO14 was replaced with the corresponding *Avr*II/*Not*I restriction fragment from pMP38/1.5 to generate pSYCO20. The *Hind*III restriction fragment containing the orf operon followed by GI P1.5 from pSYCO19 was subcloned at *Hind*III in pMP38 to generate pSYCO26. The plasmids pSYCO13 and pSYCO19 are identical except for the specific GI promoter mutant used to express glycerol dehydratase, which is P1.6 and P1.5, respectively. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of glycerol dehydratase expression level on catalyst performance metrics such as titer, rate (Table 4), yield or B12 utilization.

Table 4

	relative	3G rate (g/tank/hr)			
	dehydratase	2 mg B12	4 mg B12	8 mg B12	16 mg B12
pSYCO13	1.0	2.9	11.4	37.1	35.1
pSYCO19	0.25	11.1	20.7	32.0	33.9

EXAMPLE
Use of SYCO to examine effect of glycerol dehydratase expression level in the absence of *orfY* expression on catalyst performance

The *Avr*II/*Not*I restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO15 was replaced with the corresponding *Avr*II/*Not*I restriction fragment from pMP38/1.5 to generate pSYCO21. The *Avr*II/*Not*I restriction fragment containing GI P1.6 and 5' *dhaB1* in pSYCO16 was replaced with the corresponding *Avr*II/*Not*I restriction fragment from pMP38/1.5 to generate pSYCO22. The *Avr*II/*Not*I restriction fragment containing GI

P1.6 and 5' *dhaB1* in pSYCO17 was replaced with the corresponding *AvrII/NotI* restriction fragment from pMP38/1.5 to generate pSYCO23. The plasmids pSYCO15 and pSYCO21 are identical except for the specific GI promoter mutant used to express glycerol dehydratase, which is P1.6 and P1.5, respectively. Therefore, when combined with the glycerol pathway on pAH48 (described elsewhere) these plasmids can be used to examine the effect of glycerol dehydratase expression level in the absence of *orfY* expression on catalyst performance metrics such as titer (Table 5), rate, yield or B12 utilization.

Table 5

	relative dehydratase	3G titer (g/L)
pSYCO15	1.0	122
pSYCO21	0.25	105

EXAMPLE

Use of SYCO to examine effect of *orfW* expression on catalyst performance

The plasmids pSYCO106 and pSYCO109 are identical except that pSYCO109 does not express *orfW*. Therefore, these plasmids can be used to examine the effect *orfW* expression on catalyst performance metrics such as titer, rate, yield (Table 6) or B12 utilization.

Table 6

	orfW expression	3G weight yield (%)
pSYCO106	yes	24
pSYCO109	no	19